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14. ABSTRACT
Chlorinated phenols are persistent chemical pollutants whose exposure has been shown to give rise to both acute and long-term health risks. Many chlorinated phenols reside in soils following treatment with pesticides or defoliants, or are produced as byproducts of paper production or incineration, where they present a danger if they enter the food supply even at the parts per trillion level. The proposed research herein addresses the urgent need to incorporate biological strategies into environmental restoration efforts (bioremediation) that focus on the catalytic degradation of chlorinated phenols such as 2,4-dichlorophenol and 2,4,5-trichlorophenol (which are degradation

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Report Title

Final Report: Comparison of Natural and Engineered Chlorophenol Bioremediation Enzymes

ABSTRACT

Chlorinated phenols are persistent chemical pollutants whose exposure has been shown to give rise to both acute and long-term health risks. Many chlorinated phenols reside in soils following treatment with pesticides or defoliants, or are produced as byproducts of paper production or incineration, where they present a danger if they enter the food supply even at the parts per trillion level. The proposed research herein addresses the urgent need to incorporate biological strategies into environmental restoration efforts (bioremediation) that focus on the catalytic degradation of chlorinated phenols such as 2,4-dichlorophenol and 2,4,5-trichlorophenol (which are degradation products of Agent Orange). By focusing on enzymes as bioremediation catalysts, the proposed effort may lead to the development of novel proteins capable of the catalytic degradation of many chlorinated phenols, thereby minimizing their deleterious effects on the environment. Herein, the kinetics, spectroscopy, structure, and engineering of dehaloperoxidase (DHP) from the marine annelid Amphitrite ornata have been investigated as the focal point of such a strategy. Employing natural enzymes, or systems derived therefrom, to degrade these chlorinated or halogenated waste materials has an enormous potential to positively impact the environment and human health, and will contribute to the development of a new bioremediation industry.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

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Received	Paper
01/17/2012 14.00	Hanna Gracz, Vesna de Serrano, Matthew K. Thompson, Reza A. Ghiladi, Edmond F. Bowden, Stefan Franzen, Edward L. D'Antonio, Jennifer D'Antonio. Functional Consequences of the Creation of an Asp-His-Fe Triad in a 3/3 Globin, Biochemistry, (11 2011): 0. doi: 10.1021/bi201368u
01/17/2012 16.00	Stefan Franzen, Matthew K. Thompson, Reza A. Ghiladi. The dehaloperoxidase paradox, Biochimica et Biophysica Acta, (01 2012): 0. doi: 10.1016/j.bbapap.2011.12.008
01/17/2012 15.00	Benjamin Batternberg, Stefan Franzen, Bradley E. Stugeon, Blake J. Lyon, Hanna S. Gracz, Koroush Sasan, Rania Dumarieh, Reza A. Ghiladi. Non-Photochemical Base-Catalyzed Hydroxylation of 2,6-Dichloroquinone by H2O2 Occurs by a Radical Mechanism, The Journal of Physical Chemistry B, (12 2011): 0. doi: 10.1021/jp208536x
02/25/2015 39.00	Jing Zhao, Junjie Zhao, Stefan Franzen. The Regulatory Implications of Hydroquinone for the Multifunctional Enzyme Dehaloperoxidase-Hemoglobin from, The Journal of Physical Chemistry B, (11 2013): 14615. doi: 10.1021/jp407663n
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02/25/2015 43.00	David A. Barrios, Jennifer D'Antonio, Nikolette L. McCombs, Jing Zhao, Stefan Franzen, Andreas C. Schmidt, Leslie A. Sombers, Reza A. Ghiladi. Peroxygenase and Oxidase Activities of Dehaloperoxidase-Hemoglobin from, Journal of the American Chemical Society, (06 2014): 7914. doi: 10.1021/ja500293c
02/25/2015 42.00	Peter Le, Jing Zhao, Stefan Franzen. Correlation of Heme Binding Affinity and Enzyme Kinetics of Dehaloperoxidase, Biochemistry, (11 2014): 6863. doi: 10.1021/bi5005975
02/25/2015 41.00	Junjie Zhao, Vesna de Serrano, Stefan Franzen. A Model for the Flexibility of the Distal Histidine in Dehaloperoxidase-Hemoglobin A Based on X-ray Crystal Structures of the Carbon Monoxide Adduct, Biochemistry, (04 2014): 2474. doi: 10.1021/bi5001905
02/25/2015 40.00	Junjie Zhao, Vukica Srajer, Stefan Franzen. Functional Consequences of the Open Distal Pocket of Dehaloperoxidase-Hemoglobin Observed by Time-Resolved X-ray Crystallography, Biochemistry, (11 2013): 7943. doi: 10.1021/bi401118q
08/17/2011 3.00	Matthew K. Thompson, Stefan Franzen, Michael F. Davis, Ryan C. Oliver, Joanna K. Krueger. Dehaloperoxidase-Hemoglobin from Amphitrite ornata Is Primarily a Monomer in Solution, The Journal of Physical Chemistry B, (04 2011): 4266. doi: 10.1021/jp201156r
08/17/2011 4.00	Jennifer D'Antonio, Reza Ghiladi. Reactivity of Deoxy- and Oxyferrous Dehaloperoxidase B from Amphitrite ornata: Identification of Compound II and Its Ferrous–Hydroperoxide Precursor, Biochemistry, (05 2011): 5999. doi:

10/31/2013 36.00 Shu Jiang, Iain Wright, Paul Swartz, Stefan Franzen. The role of T56 in controlling the flexibility of the distal histidine in dehaloperoxidase-hemoglobin from Amphitrite ornata,
Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, (10 2013): 2020. doi: 10.1016/j.bbapap.2013.06.005

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The Journal of Physical Chemistry B, (10 2012): 12065. doi: 10.1021/jp300014b

10/31/2013 32.00 Stefan Franzen, Gal Schkolnik, Tillmann Utesch, Junjie Zhao, Shu Jiang, Matthew K. Thompson, Maria-Andrea Mroginski, Peter Hildebrandt. Catalytic efficiency of dehaloperoxidase A is controlled by electrostatics – application of the vibrational Stark effect to understand enzyme kinetics,
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10/31/2013 33.00 Ashlee Plummer, Matthew K. Thompson, Stefan Franzen. Role of Polarity of the Distal Pocket in the Control of Inhibitor Binding in Dehaloperoxidase-Hemoglobin,
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The Journal of Physical Chemistry B, (07 2013): 8301. doi: 10.1021/jp3116353

11/05/2012 17.00 Matthew K. Thompson, Lukasz Szatkowski, Rafal Kaminski, Stefan Franzen, Agnieszka Dybala-Defratyka. Oxidative dechlorination of halogenated phenols catalyzed by two distinct enzymes: Horseradish peroxidase and dehaloperoxidase,
Archives of Biochemistry and Biophysics, (01 2011): 22. doi: 10.1016/j.abb.2010.09.018

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The Journal of Physical Chemistry B, (04 2011): 4266. doi: 10.1021/jp201156r

11/05/2012 19.00 Francesco P. Nicoletti, Matthew K. Thompson, Stefan Franzen, Giulietta Smulevich. Degradation of sulfide by dehaloperoxidase-hemoglobin from Amphitrite ornata,
JBIC Journal of Biological Inorganic Chemistry, (02 2011): 611. doi: 10.1007/s00775-011-0762-2

11/05/2012 20.00 Bradley E. Sturgeon, Benjamin J. Battenburg, Blake J. Lyon, Stefan Franzen. Revisiting the Peroxidase Oxidation of 2,4,6-Trihalophenols: ESR Detection of Radical Intermediates,
Chemical Research in Toxicology, (11 2011): 1862. doi: 10.1021/tx200215r

11/05/2012 21.00 Edward L. D'Antonio, Edmond F. Bowden, Stefan Franzen. Thin-layer spectroelectrochemistry of the Fe (III)/Fe(II) redox reaction of dehaloperoxidase-hemoglobin,
Journal of Electroanalytical Chemistry, (03 2012): 37. doi: 10.1016/j.jelechem.2011.12.015

11/05/2012 22.00 Vesna de Serrano, Stefan Franzen. Structural evidence for stabilization of inhibitor binding by a protein cavity in the dehaloperoxidase-hemoglobin from Amphitrite ornata,
Biopolymers, (05 2012): 27. doi: 10.1002/bip.21674

11/05/2012 23.00 Stefan Franzen, Koroush Sasan, Bradley E. Sturgeon, Blake J. Lyon, Benjamin J. Battenburg, Hanna Gracz, Rania Dumariah, Reza Ghiladi. Nonphotochemical Base-Catalyzed Hydroxylation of 2,6-Dichloroquinone by H2O2 Occurs by a Radical Mechanism,
The Journal of Physical Chemistry B, (02 2012): 1666. doi: 10.1021/jp208536x

11/05/2012 25.00 Junjie Zhao, Jennifer Rowe, Jocelyn Franzen, Chi He, Stefan Franzen. Study of the electrostatic effects of mutations on the surface of dehaloperoxidase-hemoglobin A,
Biochemical and Biophysical Research Communications, (04 2012): 733. doi: 10.1016/j.bbrc.2012.03.053

11/05/2012 26.00 Junjie Zhao, Vesna de Serrano, Rania Dumarieh, Matt Thompson, Reza A. Ghiladi, Stefan Franzen. The Role of the Distal Histidine in H2O2 Activation and Heme Protection in both Peroxidase and Globin Functions,
The Journal of Physical Chemistry B, (10 2012): 12065. doi: 10.1021/jp300014b

11/05/2012 27.00 Jennifer D'Antonio, Reza A. Ghiladi. Reactivity of Deoxy- and Oxyferrous Dehaloperoxidase B from Amphitrite ornata: Identification of Compound II and Its Ferrous–Hydroperoxide Precursor,
Biochemistry, (07 2011): 5999. doi: 10.1021/bi200311u

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Received Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Franzen, S.; Zhao, J.; Ghiladi, R. "Multifunctional dehaloperoxidase-hemoglobin: how many functions can one protein have?" Oral presentation at the 8th International Conference on Porhyrins and Phthalocynanines (ICPP-8) Istanbul, Turkey, June 22-27, 2014.

R. A. Ghiladi, and Nikolette L. McCombs "Peroxygenase and Oxidase Activities of Dehaloperoxidase-Hemoglobin from Amphitrite ornata" Poster presentation at the Experimental Biology 2014 Meeting, San Diego, CA, April 26-30, 2014.

N. L. McCombs and R. A. Ghiladi "Peroxygenase and Oxidase Activities of Dehaloperoxidase-Hemoglobin from Amphitrite ornata" Oral presentation at the 111th Annual Meeting of the North Carolina Academy of Science, Raleigh, NC, March 28-29, 2014.

C. Popescu, D. Miller, D. A. Barrios and R. A. Ghiladi "Mössbauer Studies of the Ferryl, Ferrous and Ferric Dehaloperoxidase Enzyme from Amphitrite Ornata" Poster presentation at the 247th American Chemical Society National Meeting, Dallas, TX, March 16-20, 2014.

Zhao, J.; Xue, M. J.; Gracz, H.; Franzen, S. "Self-Assembley of Dehaloperoxidase-Hemoglobin Probed by Backbone Dynamics using NMR Relaxation Experiments and Molecular Dynamics Simulation" Poster presentation at the 58th Annual Meeting of the Biophysical-Society, San Francisco, CA, Feb 15-19, 2014.

Franzen, S.; Zhao, J.; Gracz, H. "Dynamics of Multifunctional Dehaloperoxidase Hemoglobin" Poster presentation at the 58th Annual Meeting of the Biophysical-Society, San Francisco, CA, Feb 15-19, 2014.

R. A. Ghiladi, and Nikolette L. McCombs "Peroxygenase and Oxidase Activities of Dehaloperoxidase-Hemoglobin from Amphitrite ornata" Poster presentation at the Metals in Biology Gordon Research Conference, Ventura, CA, January 26-31, 2014.

N. L. McCombs and R. A. Ghiladi "Peroxygenase activity of dehaloperoxidase from Amphitrite ornata" Oral presentation at the 65th Southeastern Regional Meeting of the American Chemical Society, Atlanta, GA, November 12-16, 2013.

J. Birabahara, D. Miller, R. A. Ghiladi and C. Popescu "Spectroscopic studies of the enzyme dehaloperoxidase from Amphitrite ornata" Poster presentation at the 16th Annual Undergraduate Research Symposium in the Chemical and Biological Sciences, Baltimore, MD, October 16, 2013.

Number of Presentations: 9.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received		Paper
10/12/2011	5.00	Junjie Zhao, Vesna de Serrano, Rania Dumarieh, Matt Thompson, Stefan Franzen. Effect of H55D Mutation on Kinetics and Structure of Dehaloperoxidase-Hemoglobin A, Biophysical Society 55th Annual Meeting, Baltimore, MD March 5-9, 2011. 05-MAR-11, . : ,
10/12/2011	6.00	Stefan Franzen, Matt Thompson. Distal Histidine Flexibility as the Key to the Reactivity of Dehaloperoxidase-Hemoglobin, Biophysical Society 55th Annual Meeting, Baltimore, MD March 5-9, 2011. 05-MAR-11, . : ,
10/12/2011	7.00	Koroush Sasan, Stefan Franzen. Molecular Activation by Peroxidases, Biophysical Society 55th Annual Meeting, Baltimore, MD March 5-9, 2011. 05-MAR-11, . : ,
10/12/2011	8.00	Ashlee M. Plummer, Matthew K. Thompson, Stefan Franzen. Making Substrates Out of Inhibitors: Distal Cavity Mutations in Dehaloperoxidase from Amphitrite Ornata, Biophysical Society 55th Annual Meeting, Baltimore, MD March 5-9, 2011. 05-MAR-11, . : ,
10/12/2011	9.00	Matthew K. Thompson, Stefan Franzen, Reza A. Ghiladi, Brandon J. Reeder, Dimitri A. Svistunenko. Decay of Compound ES in Dehaloperoxidase-Hemoglobin, Biophysical Society 55th Annual Meeting, Baltimore, MD March 5-9, 2011. 05-MAR-11, . : ,
10/12/2011	11.00	Reza A. Ghiladi, Rania Dumarieh, Jennifer D'Antonio. Site(s) of Tyrosyl Radical Formation in Dehaloperoxidase A and B, Biophysical Society 55th Annual Meeting, Baltimore, MD March 5-9, 2011. 05-MAR-11, . : ,
10/12/2011	10.00	Shu Jiang, Stefan Franzen. Mutagenesis Study on the Conformation of Distal Histidine in Dehaloperoxidase-Hemoglobin, Biophysical Society 55th Annual Meeting, Baltimore, MD March 5-9, 2011. 05-MAR-11, . : ,
TOTAL:		**7**

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received		Paper
TOTAL:		

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received		Paper
05/30/2011	1.00	Jennifer D'Antonio, Reza A. Ghiladi. Reactivity of Deoxy- and Oxyferrous Dehaloperoxidase B from Amphitrite ornata: Identification of Compound II and its Ferrous-Hydroperoxide Precursor, (05 2011)
TOTAL:		**1**

Number of Manuscripts:

Books

Received	Book
TOTAL:	

Received	Book Chapter
TOTAL:	

Patents Submitted

Patents Awarded

Awards

2013 LeRoy and Elva Martin Award for Teaching Excellence (awarded to Reza A. Ghiladi)

Graduate Students

NAME	PERCENT_SUPPORTED	Discipline
Jing Zhao	1.00	
FTE Equivalent:	**1.00**	
Total Number:	**1**	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Reza A. Ghiladi	0.00	
Stefan Franzen	0.08	
FTE Equivalent:	**0.08**	
Total Number:	**2**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 0.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 0.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 0.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 0.00

Names of Personnel receiving masters degrees

NAME

Total Number:

Names of personnel receiving PHDs

NAME

Total Number:

Names of other research staff

NAME PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See Attachment

Technology Transfer

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Proposal Number: 57861LS

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Proposal Title: Comparison of Natural and Engineered Chlorophenol Bioremediation Enzymes

Report Period Begin Date: 09/01/2010
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Table of Contents

Forward

In this final report, we will present an overview of our structural, spectroscopic, and biochemical studies of the protein dehaloperoxidase (DHP) from the terebellid polychaete *Amphitrite ornata*. These fundamental studies lay the groundwork for applying DHP as a potential bioremediation system against chlorinated phenols, persistent chemical pollutants that lead to both acute and long-term health risks in humans. Our studies include detailed investigations into the mechanism of oxidative dehalogenation of chlorinated phenols by DHP when initiated from both the ferric and oxyferrous states by the addition of hydrogen peroxide. The structural and electronic properties of the heme active site will also be discussed in the context of the proximal and distal cavities and the corresponding charge relays. The question of small molecule binding will be addressed, with a particular emphasis on the inhibitor binding site and the current understanding of internal versus external substrate binding. Finally, the deactivation of DHP and its physiological role will be presented. When taken together, DHP has provided us with an example of a multi-functional protein that challenges many of the assumptions behind the structure-function correlation owing to its dual roles as an oxygen transport globin and a peroxidase. More importantly, however, these studies establish that DHP has a high potential for success for being employed as a bioremediation enzyme against chlorinated phenols.

Keywords

Dehaloperoxidase; Peroxidase; Hemoglobin; Compound I, Compound II, Compound ES; Iron(IV)-oxo; Ferryl, Tyrosyl radical

Abbreviations Used

C*c*P, cytochrome c peroxidase; Compound I, the state of enzyme with a ferryl heme ($Fe^{4+}=O^{2-}$) and a porphyrin π-cation radical; Compound II, deprotonated or protonated ferryl heme state $Fe^{4+}=O^{2-}$ or $Fe^{4+}–OH^{-}$; Compound III, oxyferrous [Fe^{2+}-O_2 or Fe^{3+}-($O_2^{\bullet -}$)] state of the enzyme; Compound ES, the state of enzyme with a ferryl heme iron [$Fe^{4+}=O^{2-}$] and an amino acid radical (usually tryptophanyl or tyrosyl); Compound RH, 'Reversible Heme' state of dehaloperoxidase, formed from in the process of Compound ES decay in the absence of substrate; DCQ, 2,6-dichloro-1,4-benzoquinone, also called dichloroquinone; DHP, dehaloperoxidase; EPR, Electron Paramagnetic Resonance; HRP, horseradish peroxidase; HS, high spin (with reference to the electron spin heme iron); KPi, potassium phosphate buffer; SVD, Singular Value Decomposition; TCP, 2,4,6-trichlorophenol; TRSSA, Tyrosyl Radical Spectra Simulation Algorithm; WT, wild type.

1. Statement of the problem studied

Chlorinated phenols are persistent chemical pollutants that arise from herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D), and industrial tanning and bleaching processes. Industrially, hypochlorous acid (bleach) is used to oxidize lignin from trees in paper manufacturing. Oxidation of chlorinated hydrocarbons from incineration also leads to formation of chlorinated phenols. 2,4,6-trichlorophenol (2,4,6-TCP) is a major pollutant from the manufacture of pesticides, paper, and disinfectants among other products. There are both acute and long-term health risks that arise from exposure to chlorinated phenols. Specifically, chlorinated phenols are the major precursors of dioxins which are among the most toxic compounds known. For this reason 2,4-dichlorophenol, which is a breakdown product of 2,4-D, is cited as one of the top 70 health risks by the EPA. The health consequences of exposure to 2,4-D and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), both of which comprise the herbicide known as Agent Orange, have been the subject of many studies. Many chlorinated phenols reside in soils following treatment with pesticides, defoliants, paper production or incineration, where they present a danger if they enter the food supply even at the parts per trillion level. Given the harm caused by them, a bioremediation strategy employing naturally occurring enzymes that are capable of the degradation of such chlorinated phenols would increase our ability to combat the threat posed by these compounds.

Peroxidases are heme enzymes that can oxidize organic substrates using H_2O_2 as the oxidant, yet their use as bioremediation enzymes has been unjustifiably ignored to date because the addition of H_2O_2 is considered a relatively harsh treatment that would compromise living cells. Thus, the aim of this proposal is to establish if a new class of peroxidase enzymes, the hemoglobin-peroxidases, are able to be used in a bioremediation strategy against chlorinated phenols. The peroxidase family is characterized by an α-helical fold, a mechanism that involves activation of bound H_2O_2, and substrate binding to permit electron transfer from the substrate to the heme. Cytochrome *c* peroxidase (CcP), the prototypical member of the peroxidase superfamily, has the α-helical fold common to many of the archetypal peroxidases. By contrast, the hemoglobin family has a different α-helical fold, which is conserved across a vast number of organisms,[1] and they lack the crucial conserved amino acids of the C*c*P family that are relevant for catalysis and consequently are poor peroxidases. Nonetheless, the hemoglobin gene is thought to be the oldest heme protein gene and it is therefore possible that hemoglobins have had peroxidase activity at some point in evolution. Given the prevalence of hemoglobins, it is therefore possible some hemoglobins in modern ecosystems have peroxidase activity either preserved as one branch of the divergent evolution of globins, or perhaps as a result of convergent evolution in response to the particular evolutionary pressure of an ecosystem. These issues have become sufficiently compelling because of the discovery of the hemoglobin-dehaloperoxidase (DHP)[2] from *Amphitrite ornata* that possesses both oxygen-transport and peroxidase activities, the latter being capable of the oxidative dehalogenation of mono, di, and tri-chlorinated phenols. This final report will be devoted to our fundamental studies of the mechanism of function of dehaloperoxidase as a potential bioremediation enzyme against such chlorinated phenols. Although DHP serves a singular example, it is believed that DHP represents a possible set of mechanisms in globins that may be found in other organisms as well.

1.1 Physiological Roles of DHP

The discovery of peroxidase activity in a hemoglobin is not a complete surprise. Since the work of George and Irvine in 1951,[3-5] it has been known that hemoglobins have some peroxidase activity,[6-9] most often manifesting under pathological (post-hemolytic) conditions. The idea of engineering a peroxidase function into a myoglobin has resulted in several successful examples of mutant globins with modest peroxidase activity.[10-12] However, for the most part hemoglobins are poor peroxidases as the erythrocyte/myocyte actively suppresses their inherent peroxidase activity.[13] The evolved hemoglobins in higher life forms do not require peroxidase activity and, indeed, there is a conflict between H_2O_2-binding and activation with the binding and transport of

oxygen. These studies cause us to ask: “When is peroxidase activity truly important for physiology?” The results discussed below in this final report reveal highly specific aspects of the mechanism and interactions of both substrates and hydrogen peroxide with DHP that clearly show that peroxidase activity is not an artifact in this case.

The consideration of the environmental factors and complex interactions of a number of molecules with DHP suggest that there is an evolutionary pressure on DHP that is consistent with its current place as the best peroxidase of any known globin. *A. ornata* is a worm found in relatively shallow muddy coastal waters and marine estuaries. There are many life forms in these ecosystems that produce toxic brominated and chlorinated compounds.[14-17] Such compounds are repellents that offer protection against predators and may have other functions in preventing attack by fungi or microorganisms. In North Carolina, some of the coastal regions have such a high load of these odiferous brominated compounds that they are called "bromine flats". A number of species in these ecosystems produce brominated phenols, pyrroles and indoles as the most common compounds. Our hypothesis is that DHP has evolved to degrade a number of these compounds, which are produced by other organisms and indeed by related heme proteins.[18] DHP is a sufficiently active peroxidase that it was originally named "dehaloperoxidase" when it was discovered in 1996 as the fraction from *A. ornata* that was capable of degrading the known marine toxin 2,4,6-tribromophenol.[2] It is remarkable that DHP was known previously as a hemoglobin, and was characterized first as such in 1977 by Bonaventura et al.[19] The fact that DHP is a globin became obvious when the DHP structure and amino acid sequence were determined.[20,21] Given what is known about the role played by *A. ornata* in benthic ecosystems, DHP may well be one of the few proteins capable of protecting the worm (technically a terebellid polychaete) against the onslaught of the toxic compounds produced by many of its neighbors.[14,15,22,23] Given this history and dual roles in biological function, the name hemoglobin-dehaloperoxidase will be used in this final report in addition to the more commonly used shorthand notation of dehaloperoxidase (or DHP).

1.2 The Two Isoforms of DHP

There are two isoforms of DHP, known as A and B.[22] The reactivity of DHP A as a peroxidase was shown first in 1996.[2,24] In 2010, recombinant DHP B was created by mutation of the 5 amino acids that differ from DHP A.[25,26] DHP B has four times higher peroxidase activity than DHP A for 2,4,6-tribromophenol, which makes this isoform the naturally occurring hemoglobin with greatest known peroxidase activity. DHP is the most abundant protein in *A. ornata* and is responsible for the reddish color of the organism; however, it is not known what DHP percentage is produced in each isoform, if their expression is environmentally controlled, and how their concentrations and distribution compares to the erythrocruorins. The available evidence suggests DHP A is the dominant form in DHP isolates from *A. ornata*.[21] The isoforms are of particular interest mainly because they are examples of multi-functional proteins that permit one to begin to study the structural origins of the unique reactivity of this small protein. There are two forms of hemoglobin in the organism: DHP that is found in the coelom (space between the outer wall of the worm and its feeding tube), and a giant hemoglobin that is found in the tentacles. The oxygen affinities: 4.2 torr for DHP A, 2.0 torr for DHP B[27] and 11 torr for giant hemoglobin[19] suggest that the worm tentacles absorb oxygen with the giant hemoglobin (or erythrocruorin), which is then transferred to DHP for storage and release in the main body.[28,29] Unlike the giant Hb, DHP is a monomeric intracellular protein.[19] There is some tendency for DHP to dimerize in vitro[30] but since its intracellular concentration is not known, the DHP physiological state is uncertain. The dimer interface observed in DHP crystals is different from those in mammalian hemoglobins.[21] Nonetheless, DHP A and B may be constituents of the erythrocruorin since there are only two hemoglobin genes identified in *A. ornata*.[22]

While this final report will focus mainly on the dehaloperoxidase activity of DHP, the multi-functional nature of the protein is noteworthy. It has recently been shown that DHP can support both peroxygenase and oxidase mechanisms for certain substrates.[31] DHP is also known

as an excellent sulfide oxidase,[32] which is yet another function related to the proposed protective function. The available structural evidence suggests that there are at least three binding sites (two internal and one external) that can account for this variety of mechanisms and specific inhibition. While the multi-functional nature of DHP is of great interest, this final report will focus specifically on a comparison of the peroxidase activity in DHP with that in prototypical peroxidases, such as horseradish peroxidase (HRP), which was the main focus of the original proposal.

2. Summary of the most important results

2.1 Peroxidase Mechanism of DHP

2.1.1 DHP Mechanism Initiated from the Ferric State

Both isoenzymes of DHP (A & B) catalyze the oxidative degradation of 2,4,6-trihalogenated phenols (substrate) to the corresponding 2,6-dihalo-1,4-benzo- quinones in the presence of H_2O_2. Several recent studies have focused on the characterization of DHP, as well as elucidating the mechanism of this reaction when starting from the ferric resting state. In the absence of a reducing substrate, DHP initially reacts with H_2O_2 to generate Compound I, which rapidly converts (<1 ms) to an iron(IV)-oxo heme center with tyrosyl radical that has been termed Compound ES by analogy with cytochrome *c* peroxidase (Figure 1). The role(s) for the radical(s) in Compound ES with respect to protecting the enzyme against heme bleaching is discussed in a later section. The catalytic competency of both Compound I and Compound ES intermediates in oxidizing the substrate 2,4,6-tricholorophenol (TCP) to 2,4-dichloroquinone (DCQ) has been shown. As discussed in a later section, it has also been found that in the absence of substrate, there is the formation of a new species named Compound RH,[33] which is unique to dehaloperoxidase and has not been found in any other globin. This species is thought to protect the enzyme from oxidative damage and irreversible inactivation under conditions of low substrate concentration. The turnover of TCP by horseradish peroxidase (HRP) and *C. fumago* chloroperoxidase have been studied previously.[34,35] While both are more rapid than DHP A by a factor of ~12, the catalytic activity of DHP A is at least 10 times greater than that of SWMb.[36,37] DHP B is ~4.3-fold more active than DHP A, so that it is within a factor of 3 of HRP activity.[25,38] The dehaloperoxidase activity of DHP has also been reported to be greater using meta-chloroperoxybenzoic acid in place of hydrogen peroxidase as the oxygen donor to initiate the reaction.[39]

The evidence strongly suggests an overall two-electron oxidation of TCP by DHP that proceeds through discrete one-electron steps. The product DCQ itself is not an innocent species, having been shown to react separately with both Compound ES and ferric DHP to yield oxyferrous DHP in either case.[25,33] The unusually high reduction potential for DHP (when compared with other peroxidases) of ~+205 mV reported for DHP likely facilitates reactions with DCQ that ultimately favor the reduction of the heme prosthetic group and formation of oxyferrous DHP.[40] Thus, DCQ chemistry may represent one possible link between the two namesake activities of hemoglobin-dehaloperoxidase by allowing for the ferric state to either initiate a peroxidase pathway in the presence of TCP and H_2O_2, or form the oxyferrous complex in the presence of DCQ (itself generated from the aforementioned peroxidase pathway) and thus enabling the O_2-transport function.

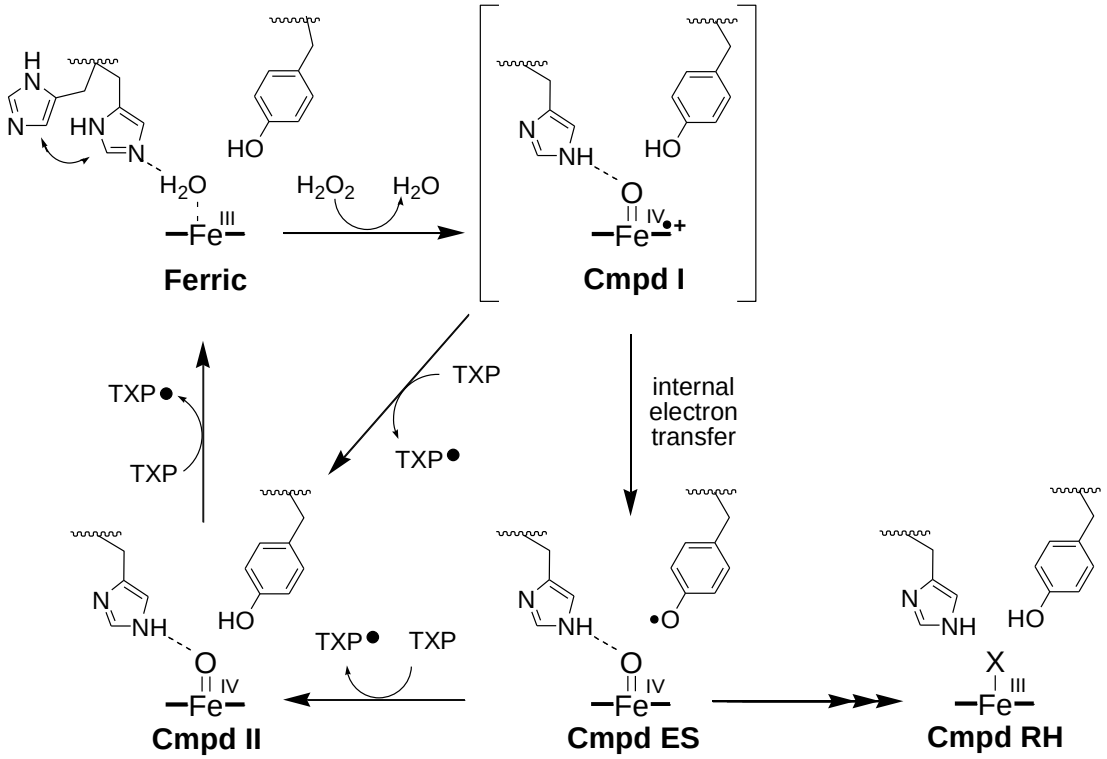

Figure 1. Mechanism for trihalophenol (TXP) oxidation by ferric DHP utilizing H_2O_2 as the oxidant.

2.1.2 DHP Mechanism Initiated from the Ferrous State

While progress has been made toward understanding the DCQ-driven functional switch of how the peroxidase-active ferric DHP state can be converted to the oxygen-binding ferrous form, the reverse, wherein oxyferrous DHP is activated toward peroxidase function, is more controversial. Given that reversible oxygen-binding is only mediated via a ferrous heme in globins, and that peroxidase activity is initiated from ferric centers and to the exclusion of the oxyferrous oxidation state from the peroxidase cycle, the bifunctional nature of DHP as a globin-peroxidase appears to be at odds with the traditional starting oxidation state for each individual activity – this has been referred to as "the dehaloperoxidase paradox".[41] In a peroxidase reaction that is unique to DHP, it has recently been reported that dehaloperoxidase activity has been observed when the catalytic cycle is initiated from the oxyferrous state, but that the catalytically active species is only formed in the presence of the TCP substrate.[42] The lack of reactivity observed for oxyferrous DHP when trihalophenol substrate was absent may have a physiological role, preventing formation of Compound RH (with its attenuated dehaloperoxidase activity) when exposed to hydrogen peroxide for short reaction times (< 60 s), and also minimizing the deleterious effects of unwanted peroxidase activity in the absence of a reducing substrate. These observations suggest that the involvement of substrate is the key factor in the functional switch of DHP from that of an oxygen-binding protein to a peroxidase. The two current hypotheses for how trihalophenol substrate is involved in the activation of oxyferrous DHP for peroxidase function are:

i) TCP radicals (TCP•), themselves formed from trace amounts of the ferric enzyme reacting with TCP and hydrogen peroxide, oxidize oxyferrous DHP to the peroxidase active ferric form. This radical-based activation mechanism has precedent with the monofunctional lignin peroxidase (LiP), where veratryl alcohol oxidation has been shown to yield radical species that oxidize oxy-LiP to the peroxidase-active ferric enzyme. Evidence in support of this substrate radical-based activation include: (a) the lag time seen of the dehaloperoxidase activity when turnover is initiated by H_2O_2 addition to the TCP-bound oxyferrous enzyme is sensitive to both TCP and H_2O_2 concentration showing that both substrate and H_2O_2 are necessary to convert the

enzyme to the catalytically active state. The lag time also is decreased when a small amount of ferric DHP is added to oxyferrous DHP to purposefully generate TCP• following addition of H_2O_2.[43] (b) In the presence of excess TCP, one equivalent of H_2O_2 (or even as little as 0.5 equiv) converts oxyferrous DHP to the catalytically active ferric state.[43] (c) Ferrocyanide, a known peroxidase substrate with a structure that is completely different from TCP, also triggers the functional switch of oxyferrous DHP to catalytically active enzyme showing that an inorganic one-electron redox agent can take the place of the TCP• in promoting the switch.[44] (d) Ferric heme iron ligands such as imidazole and cyanide and organic radical traps such as 5,5-dimethyl-1-pyrroline-N- oxide (DMPO) effectively inhibit the TCP-based switch, although DMPO does not inhibit the ferrocyanide-based switch which does not involve organic radicals.[44] Taken together, these data support a mechanism for the activation of oxyferrous DHP in which trace amounts of the ferric enzyme present in the oxyferrous protein catalyze formation of TCP• which oxidizes deoxyferrous DHP to the catalytically active ferric state.

ii) Substrate binding to DHP destabilizes the oxyferrous state in the presence of hydrogen peroxide, leading to a reaction between ferrous DHP and H_2O_2.[42] Evidence in support of this ferrous DHP based reaction include: a) anaerobic studies on the reaction of deoxyferrous DHP with H_2O_2 yielded Compound II, one of the active species of the peroxidase cycle, and did not require substrate to be present. The conversion of deoxyferrous hemoproteins to Compound II has been noted and/or postulated in a number of other systems, including horseradish peroxidase, leghemoglobin, lactoperoxidase, myeloperoxidase, cytochrome *c* peroxidase and KatG. The reaction of oxyferrous DHP with H_2O_2 in the presence of as little as one equivalent of TCP co-substrate also identified DHP Compound II as the first intermediate formed. This result demonstrated that the activation of oxyferrous DHP by trihalophenols can lead directly to a peroxidase-active form of the enzyme without first invoking a ferric resting state. Furthermore, the importance of this observation is also underscored by the *in vivo* role of dehaloperoxidase as an oxygen-transport protein, wherein the globin exists in both the oxy- and deoxyferrous states, and it is reasonable to surmise that the initiation of a peroxidase pathway should not be limited to only one of the two major states of a globin.

Given that there is significant evidence in favor of each of the above two hypotheses, it is plausible that more than one mechanism of activation may be present in DHP.

2.2 Structural relationship of DHP with peroxidases

Dehaloperoxidase has a globin fold. Although its sequence identity with known globins is low, it has high structural similarity. The spectroscopic observables of DHP are likewise similar to globins. These similarities are so pronounced that there was a concern for many years that DHP was not truly an enzyme, but rather there was an impurity present or some other explanation for the apparent peroxidase activity. The cloning of DHP and careful study of its kinetics have both allayed these concerns. Now the question that arises is how DHP is capable of carrying out peroxidase chemistry, when it has so little structural similarity with peroxidases.

The classic peroxidase structural features have been described in terms of a "push-pull" mechanism leading to activation of bound H_2O_2. The push on the proximal side is due to a charge relay that increases the basicity of the ligand to the heme Fe. This in turn supports the oxidation state of the Fe needed for the active species Compound I and II. The pull is due to hydrogen bonding on the distal side. This feature of peroxidases is crucial since the pull is responsible for heterolytic bond cleavage. Globins and peroxidases both have one proximal and one distal histidine. Yet, the function of these amino acids differs significantly in these two classes of proteins. In the following we will discuss the aspects of polarization and conformation of these respective peroxidases in DHP, with reference to the congeners in hemoglobin (myoglobin) and peroxidases. In order to understand these two aspects of peroxidase function, we consider the structural aspects of the proximal and distal sides of the protein in separate sections.

2.2.1 Structural and electronic properties of the proximal side of the heme

The "push" on the proximal side is facilitated by an increase in the partial negative charge on the proximal histidine due to a charge relay. Goodin et al. first proposed the analogy between the Asp-His-Fe charge relay of peroxidases and Asp-His-Ser charge relay in serine proteases.[45] The charge relay in serine proteases consists of a strong hydrogen bond between Asp and the Nδ of His, which leads to partial deprotonation and an increase of the negative charge of the His. The partial negative charge is communicated to the Ser through a second hydrogen bond. The partially deprotonated Ser is a good nucleophile, which is the essential feature of a serine protease. The analogous charge relay in peroxidases increases the charge density on the heme. Thus, the functional aspect of the charge relay is to alter the properties of the proximal histidine so that the heme Fe can support the Fe(IV) oxidation state required for the formation of Compounds I and II. For example, in C*c*P this relay is formed by Asp235-His175-Fe. Mutation of Asp235 to Asn causes a dramatic reduction in C*c*P enzymatic activity, which correlates with a shift in the frequency of the Fe-His resonance Raman band. In myoglobin, the His93-Ser92 hydrogen bond has been studied in a series of mutants,[46,47] including the S92D variant that would mimic the Asp-His-Fe charge relay.[48] However, it was concluded that this hydrogen bond in myoglobin is responsible for maintaining the protein structure of the proximal heme pocket, but that it does not strongly affect the electronic structure of the heme nor that of the His93 imidazole ring.

DHP lacks the typical peroxidase proximal charge relay. Instead, DHP has a strong hydrogen bond between the backbone carbonyl of L83 and the Nδ-H of the proximal histidine (H89). Can such a hydrogen bond take the place of the Asp-His-Fe charge relay? Although a hydrogen bond of this type can polarize the histidine to an extent, the fact that the backbone carbonyl is neutral limits the possible extent of charge displacement.[49] The effect of the charge relay has been studied using density functional theory (DFT) methods, which show that all neutral hydrogen bonding interactions are qualitatively different from the anionic interaction formed by the Asp in C*c*P.[50] Thus, the charge relay appears to be missing in DHP. Evidence for this can be found in resonance Raman spectroscopy of the proximal His stretching vibration as the value of the wavenumber of the Fe-His band scales with the extent of charge transfer to the Fe. The polarization tends to result in a greater force constant for the Fe-His stretch. The values for typical globins are in the range ~220 cm^{-1} and for peroxidases they are ~242 cm^{-1}. DHP is exactly in the middle of these two values at 230 cm^{-1}.[51] This observation is consistent with the placement of DHP in an intermediate range having a weak polarization, but nonetheless, one that is outside the range of other globins.

In order to further explore the implications of the proximal hydrogen bonding in DHP, two mutants were constructed to introduce a charge relay into DHP. The M86D and M86E mutants were designed to permit the Asp (D) or Glu (E), respectively, amino acid to interact with the proximal histidine and thereby give rise to a charge relay. However, buried charges are not particularly stable and the X-ray crystal structure of the M86E (PDB 3MYM) DHP mutant revealed that the Glu (E) side chain had rotated out into the solvent in order to avoid being buried inside the protein. Table 1 provides a complete list of the PDB structures of DHP. Figure 2 shows molecular dynamics (MD) simulations of both the M86D and M86E mutants based on the 3MYN and 3MYM X-ray crystal structures, respectively.[52] These simulations show that M86D can interact with H89 in a dynamic fashion, although neither of the negative amino acids has the same strong effect as the Asp in C*c*P. While the M86E mutant completely failed to give rise to a charge relay, the M86D mutant did have some degree of polarization of the histidine based on ^{13}C-^{15}N NMR spectrum, resonance Raman spectroscopy, and the reduction potential.[52] In the final analysis, the charge relay in DHP actually reduced the activity of the enzyme. This is believed to be due to the increased tendency to form hemichrome (a low-spin globin state seen en route to denaturation). The conclusion of the studies of the proximal mutants in DHP was that there is a balance of factors such that there is an optimum polarization of the heme Fe. An increase in polarization enhances reactivity, increases the binding of H_2O_2, and stabilizes the higher oxidation state of the heme Fe. Up to a point the increase in polarization is completely favorable for

peroxidase activity since it permits the formation of Compound I. If the extent of polarization is too great, then there will be a greater tendency to form the six-coordinate hemichrome and deactivate the enzyme. Du et al. reported that the M86E DHP variant has 9-fold higher activity than wild-type DHP at pH 7 and 1.4-fold higher activity at pH 5.4.[53] However, the activity of the mutants is complicated by the fact that k_{cat} and K_m in the Michaelis-Menten kinetic scheme[54] appear to be affected in opposing ways. D'Antonio et al. reported that k_{cat} for M86D and M86E are increased by factors of 2.1 and 1.6, respectively, relative to the wild type.[52] This is consistent with the greater negative charge near the Nδ-H atom, which enhances the charge relay effect at the heme Fe. However, the effect on K_m was opposite such that the M86D and M86E increased by factors of ~9 and ~3, respectively. Therefore, the catalytic efficiency (k_{cat}/K_m) decreases by a factor of 4.2 and 1.7 relative to wild type for M86D and M86E, respectively. The effect on K_m can be interpreted as a decrease in the on rate for H_2O_2, which is due to the interaction of the distal histidine with the Fe in these mutants observed in the greater tendency for hemichrome formation.

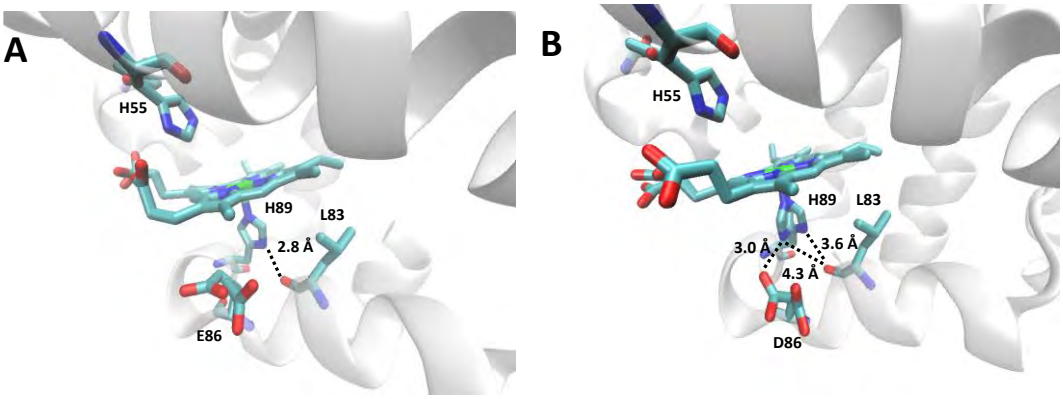

Figure 2. Coordinates of the mutated residues M86E and M86D obtained from MD simulations. The bases for the simulations were X-ray crystal structures PDB 3MYM and PDB 3MYN, respectively.

The proximal cavity mutant H89G has been made in DHP.[55] Unlike the case of SWMb where the analogous proximal cavity mutant (H93G) has been made,[56-58] it was not possible to attempt a rescue of function by addition of other ligands to DHP(H89G): this mutant was completely inactive even when imidazole was bound to the heme Fe in the proximal pocket. One can surmise that this is the case since the imidazole would likely be polarized in DHP and this polarization is difficult to replicate in the proximal cavity mutant. For example, the analogous mutant C*c*P H175G is also inactive.[59,60] Unlike the case of SWMb where the analogous proximal cavity mutant (H93G) has been made, it was not possible to rescue the function of DHP(H89G) by addition of imidazole or other small molecules.[56-58]

2.2.2 Structural and electronic properties of the distal side of the heme

Peroxidases employ the distal histidine as a key acid-base catalyst that facilitates heterolytic O-O bond cleavage as described by the Poulos-Kraut mechanism.[61] In all peroxidases, the distal histidine is assisted by an arginine, which is thought to permit stronger hydrogen bonding than would be possible for histidine alone. In DHP, however, the distal side lacks such an arginine, and moreover the distal histidine has no other near neighbors capable of enhancing the hydrogen bond strength. Instead, we have noted that the distal histidine of DHP is unusually flexible.

Initially, the flexibility of the distal histidine was hypothesized based on the X-ray crystal structures of DHP.[20,21,62-65] A persistent theme in the many structures of DHP in different ligation states is the observation that the histidine, H55, is observed in two conformations, one internal and one external. The internal conformation in the distal pocket is within range to hydrogen bond

to ligands bound to the heme Fe. This conformation is shown in Figure 2. The external or solvent exposed conformation is partially stabilized by interaction with the propionate side chains. This conformation is also forced by the binding of the inhibitor 4-BP as shown in Figure 4 below. The heme of DHP is ~ 1 Å more deeply buried in the globin than in other hemoglobins, which means that the propionates are positioned in an appropriate distance to hydrogen bond.[20,21] Our hypothesis states that the balance between the internal and external conformations is determined by the hydrogen bond strength. H_2O can form a strong hydrogen bond to H55, and therefore the population of DHP in the metaquo form has an internal conformation for H55.[62-64] This applies to the oxyferrous and metcyano forms. However, H55 is observed mainly in the external conformation when CO is the ligand.[66] Similar studies have been performed for the distal histidine in SWMb and other myoglobins and hemoglobins. However, in these globins the internal conformation is dominant unless the pH is lowered to pH ~4. DHP is unique in that the crystallization conditions of pH 6 are sufficient to drive the histidine to at least 40% into the external conformation.

The flexibility of the distal histidine may well be crucial for peroxidase activity. In a protein that lacks the ancillary arginine and has no other obvious amino acids that can assist in strengthening the hydrogen bonding, it is possible that H55 is susceptible to the conformation of the protein, which in turn may affect substrate and inhibitor binding. The inhibitor effect is easy to understand since it displaces H55 100% into the external conformation. However, substrate binding is more complex and is discussed further below. The flexibility of H55 plays a role in maintaining an open distal pocket, which may be necessary for entry and exit of relatively large substrate molecules. There is a simultaneous effect of substrate binding, which may result in forcing the histidine deeper into the distal pocket, thereby strengthening the hydrogen bond and permitting the acid-base catalysis necessary for the Poulos-Kraut activation of bound peroxide.[67]

One can test the role of the distal histidine by two types of site-directed mutations involving either replacement of the histidine itself (first type) or surrounding amino acids that controls its conformation (second type). First, one can replace the histidine itself with another amino acid. Thus far, the mutants H55D, H55V and H55R have been studied.[55,68] Second, one can alter the amino acids in the vicinity of H55 in order to affect its dynamics. The mutants T56G, T56A, T56S and T56V have been studied in this category.[69] Turning to the first class of mutations, it is not surprising that the effect of direct mutation of H55 is a drastic decrease in reactivity. The mutant H55V has essentially no reactivity. H55R is decreased by a factor 5 and H55D is decreased by a factor of ~10. The fact that the charged amino acids are not completely inactive suggests some small degree to which the mutated amino acids may play a role of an acid-base catalyst. H55D has been studied most extensively. In this case, the X-ray crystal structure shows that the aspartate is essentially 100% in the external conformation, which is expected for a negatively charge amino acid. However, molecular dynamics simulations suggest that Asp55 may have some tendency to enter the protein as neutral (protonated) aspartic acid.[68] This form of the amino acid may have a small degree of activity. Thus, even in a much less active amino acid we can observe that the conformational flexibility of the distal ligand is important.

The second category of mutants involves a change in the conformation of the distal histidine. It is at first surprising that the T56 mutants cause an apparent acceleration of the enzymatic rate.[69] However, this greater activity must compete with hemichrome formation, which inactivates DHP. Thus, we see that the same issue discussed for the proximal ligands reappears in the context of the distal amino acids. Pushing the protein towards a greater degree of ligation (distal) or stability of binding (proximal) can accelerate the enzymatic rate, but simultaneously increases the equilibrium for hemichrome formation, which irreversibly deactivates the protein. The evolutionary choice in each case appears to optimize the enzyme by providing a high rate of turnover with minimal hemichrome formation under native conditions.

2.3 Optimal conditions for DHP function - physiological relevance

There is a pronounced pH effect on substrate turnover that also relates to the optimal pH for substrate turnover. DHP has an unusually low pH for the acid-alkaline transition (Fe^{3+}-OH_2 -> Fe^{3+}-OH + H^+), which is the pK_a of the metaquo form. In DHP, this pK_a is 8.1.[70] Connected with this observation is the fact that the optimum pH for turnover is pH = 7.5 when the criterion used is the greatest concentration of product at the end of an assay. DHP has the unusual feature in that the initial rate accelerates as the pH is lowered below pH 7.5. Figure 3 shows that if one were to examine initial rate alone it would appear that the ideal pH is pH 5 or lower. However, this is not a true measure of substrate turnover since there is a deactivation of DHP that competes with turnover, and it is the deactivation that is observed at lower pH rather than substrate turnover. The deactivation can be observed spectroscopically and is linked to the formation of the inactive (or less active) form of DHP called Compound RH. The fact that deactivation is occurring is also evidence from the kinetic traces, which have a rapid initial onset, and followed by an inactive phase at pH 5. The origins of the branching has been studied in detail using electron paramagnetic resonance (EPR) to understand the pathways for radical formation and transfer among the various tyrosines of DHP.[71,72]

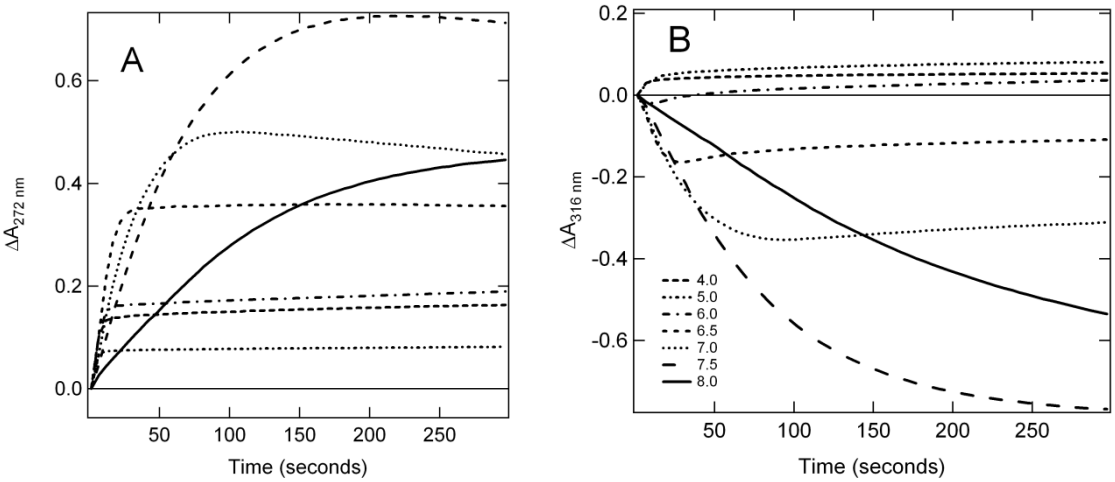

Figure 3. Single wavelength kinetics of the oxidative debromination of TBP by DHP as a function of pH. The fits to a single exponential function can be found in Ref. 73. **A.** The kinetics at 272 nm. **B.** The kinetics at 316 nm.

The apparent optimal pH of 7.5 is consistent with the observation of DHP as the most abundant protein in the coelom of *A. ornata*. DHP is an intracellular hemoglobin and, as such, it must function at the pH of cytosol, which is 7.4. Hence, if we compare DHP to the various classes of peroxidases, DHP would appear to be a class I peroxidase. Initially, DHP was studied at low pH by analogy with HRP, which is a secretory peroxidase. Both HRP and DHP are known to oxidize phenols, but the similarity may end there. Secretory peroxidases function at low pH (typically pH 5) because they are secreted into the soil where the pH is typically low. Their function is to degrade various biopolymers so that the components may be ingested by the host that secreted the peroxidase. DHP functions as a multi-functional intracellular peroxidase and as such it follows different criteria. First, it appears that different substrates bind in different modes (discussed below). The inhibitor binds in a specific binding site. If we examine the bromophenols, which are found in the ecosystems where *A. ornata* lives, we can see the interplay of pH, mode of binding and the optimal chemical change.

4-BP (and 4-CP) can dimerize and polymerize upon radical formation.[74] The reason that 4-BP is an inhibitor may be related to this fact. It is possible that this polymerization is detrimental to the organism, and therefore should be suppressed; hence, the binding of 4-BP in an internal binding site. 2,4,6-TBP, on the other hand, is much more toxic, although less abundant.[16] The oxidation of the 2,4,6-TBP yields 2,6-dibromoquinone, which is not a harmful molecule and is

even further oxidized. Thus, we can understand that 2,4,6-TBP, unlike 4-BP, is readily oxidized by a peroxidase mechanism. Less is known about 2,4-dibromophenol (2,4-DBP), but we know that it is at a minimum oxidized to a quinone in the same way that 2,4,6-TBP is.

2.4 Inhibition of DHP

The enzymatic mechanism of DHP was perhaps first truly clarified by the realization that certain phenols act as inhibitors rather than substrates. The series of 4-halophenols (4-XPs), 4-iodo-, 4-bromo-, 4-chloro-, 4-fluoro and phenol itself bind in an interior binding pocket in the distal pocket of the globin (denoted (4-IP, 4-BP, 4-CP, 4-FP and P, respectively).[65] Figure 4 shows that inhibitors bind in an orientation perpendicular to the heme Fe and slightly offset from the center of the distal pocket so that the heme Fe is partially blocked by the inhibitor.[20,21,65] This partial covering of the heme Fe can be observed by X-ray crystallography, but is also evident in the effect of 4-XP on heme-bound ligands. The binding of 4-XP competes with the binding of ligands to the heme Fe.[65] For example, a water ligand is bound to the ferric heme Fe of the normal resting DHP with approximately 40% of the population, the rest of the population being 5-coordinate.[71] These populations can be observed by resonance Raman (rR) spectroscopy. When 4-XPs bind they displace the heme-ligated water, and at sufficiently high 4-XP concentration the heme is 100% 5-coordinate. Thus, rR spectroscopy can be used to monitor the binding of the inhibitors. The inhibitors bind with decreasing affinity as the halogen radius decreases. This trend is counterintuitive if one imagines that the inhibitor must displace amino acids in order to bind in the interior of the DHP. Rather, the trend can be explained by the existence of a cavity inside the protein, which is located precisely where the para-halogen atom resides in the bound state. Figure 4 shows the binding site for Xe discovered by high pressure Xe X-ray crystallography. The Xe binding site is a cavity that is located precisely where the halogen binds in the distal pocket.[75]

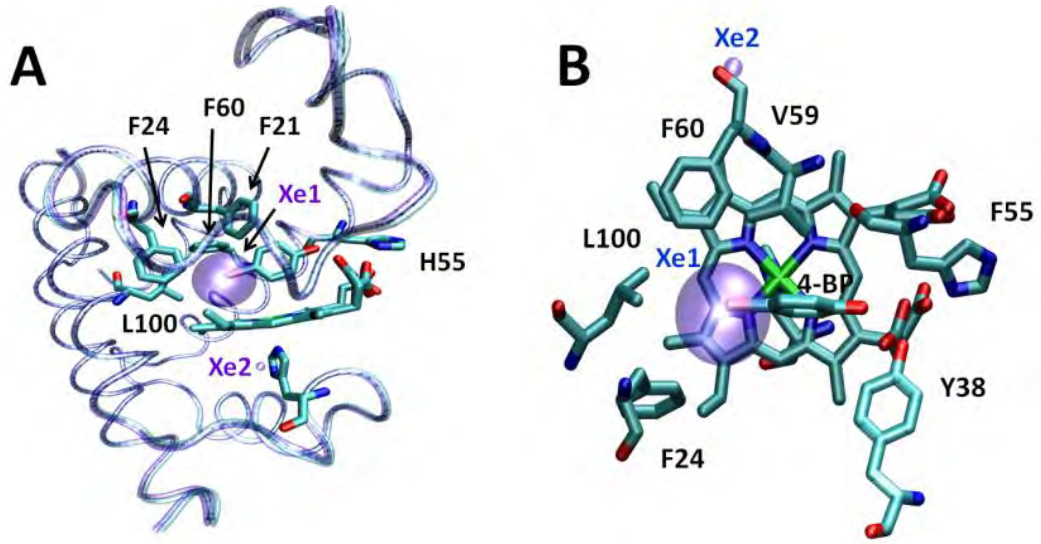

Figure 4. Overlay of two X-ray crystal structures showing the internal inhibitor binding site (PDB 3LB2) and the Xe binding site (PDB 3MOU). The Xe binding site is shown by the transparent blue sphere. The bromine atom of 4-BP is shown near the center of the sphere. The hydrophobic amino acids surrounding the Xe/Br binding site are shown. The distal histidine is forced into the solvent-exposed (exterior) conformation.

Inhibitor binding is quite specific in DHP. However, a range of molecules with similar shapes can also bind in the inhibitor binding site. Para-hydroquinone (H_2Q) is one such molecule. H_2Q binds in the inhibitor site and provides information on the role proton-coupled electron transfer in DHP. H_2Q is converted to BQ only if the distal histidine, H55 is present to act as a proton shuttle. First, H2Q can be catalytically oxidized to para-benzoquinone (BQ) in the presence of H_2O_2. In the absence of H_2O_2, H_2Q reduces the heme to the ferrous state and actively prevents turnover of substrates such as 2,4,6-TCP.[76] While the catalytic oxidization H_2Q to BQ. While this

chemical transformation is not remarkable, the fact that it occurs in the inhibitor binding site is unique. The binding of H_2Q in a site immediately adjacent to the heme Fe means that its oxidation is very rapid. Thus, if H_2Q and the substrate 2,4,6-TCP are both present, the H_2Q reaction dominates and no turnover of TCP is observed until all of the H_2Q is oxidized. This competition gives rise to a kinetic lag phase during which no turnover of 2,4,6-TCP is observed. Once all of the H_2Q has been oxidized there is a sudden onset of 2,4,6-TCP activation.[76]

2.5 The substrate binding conundrum

2.5.1 Evidence for an external binding site

The location of the substrate binding site is one of the outstanding unsolved problems in DHP research. Normally, substrate binding in peroxidases occurs at the heme edge (HRP, APX) or at an external docking site that has an electron transfer pathway to the heme (C*c*P). Since the discovery of peroxidase activity in DHP there has been an underlying assumption that DHP follows this model and, indeed, experimental data suggest that DHP has a similar external binding site. For example, flow-EPR has been used to study the formation of a semi-quinone radical in DHP.[77] When compared to HRP, which has an established external binding site near the heme edge,[78] we observe that both of them produce semi-quinone radicals in the flow EPR experiment. This suggests that both enzymes have an external binding site. If DHP follows a well-established pattern observed in heme peroxidases,[79] the external binding site would be on the heme δ-edge labeled in Figure 5. An examination of the structure shows that this location has the shortest path for electron transfer from the heme to an externally bound phenol. However, we reiterate that despite extensive studies, there is no direct evidence to date that corroborates this hypothesized binding site.

On the contrary, studies of interactions of phenolic substrates have revealed at least two different internal binding sites (discussed in section 2.5.2 and 2.5.3). It has already been established that the binding site for 4-BP is inhibitor (section 2.4) and the evidence to date suggests that the internal substrate binding sites, discussed below, are also inhibitory for peroxidase function. These sites may be observed because of other functions in DHP (such as the recently characterized peroxygenase function). However, any radical produced by one-electron oxidation would likely react before exiting from the protein, which clearly would inhibit a peroxidase function. We must also consider the fact that DHP is rather rapidly deactivated during the course of the flow-EPR experiment discussed above. This can be understood in the context of the radical pathways in the protein involving the 5 tyrosines in DHP. We conclude that the radical pathways required for an external site may simultaneously result in side reaction(s), which alter the heme and inactivate the enzyme. The exact mechanism is not known, but it is clear that the very conditions that promote peroxidase activity also tend to result in some degree of inactivation by chemical modification of the heme (as discussed in section 2.6). Members of the C*c*P family appear to have a greater resistance to the kind of inactivation observed in DHP, perhaps because of their evolutionary specialization. Despite the data showing that an external binding site exists, it is not possible to exclude an internal binding site. Indeed, the observation of an internal site for 4-BP has been one of the hallmarks of the unique role played by DHP since its discovery as a peroxidase in 1996.

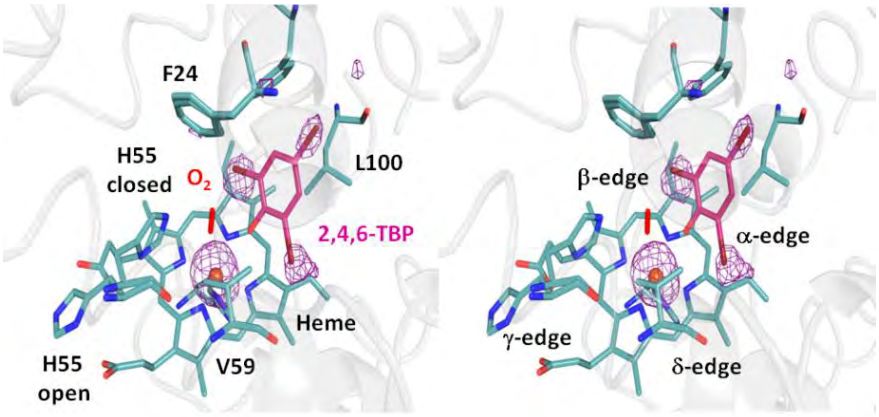

Figure 5. Stereo view of the distal side of the heme pocket of DHP A (PDB entry 4HF6), showing the position occupied by the substrate, 2,4,6-TBP (shown in magenta), at an internal binding site in back of the distal pocket. The heme is shown as are amino acid residues L100, F24, V59 and H55 in two conformations (open and closed). The substrate location was determined with the help of the anomalous difference electron density map contoured at 3σ electron density level constructed from the SAD data set at the Kα electron transition energy for Br (λ ~ 0.92 Å Br edge) relative to Fe. Anomalous density is shown in the figure for the three Br atoms and the heme Fe.

2.5.2 Evidence for TBP binding sites in the distal pocket

It is rather easy to soak the inhibitors 4-IP, 4-BP, 4-CP and 4-FP into a crystal of DHP and to obtain a crystal structure.[20,21,65] However, when the same exact experiment is attempted with 2,4,6-TCP, no substrate molecules are observed in the crystal. It was determined that a major reason for this is the relatively low solubility of 2,4,6-TCP. This is even more of a problem for 2,4,6-TBP. One can overcome this problem using non-aqueous solvents. Use of 10% methanol, 2-propanol or dimethyl sulfoxide increases the solubility of 2,4,6-TCP and 2,4,6-TBP sufficiently that one can observe a small amount of electron density in the distal pocket (PDB 4FH6, 4ILZ, and 4FH7).[76] However, the X-ray crystal structure (PDB 4FH6) shown in Figure 5 indicates that the substrate binds internally. Table 1 lists the various inhibitors and substrates that have been observed in X-ray crystal structures of DHP. Internal binding of the substrate is difficult to rationalize with the observed kinetics from the flow-EPR experiment, or in fact, any peroxidase mechanism.[77] The peroxidase mechanism commonly involves one-electron oxidation to form a radical followed by disproportionation of the radicals to form one molecule of reactant and one of the oxidized intermediate that will convert to the product upon reaction with H_2O.

2.5.3 Evidence for TCP binding sites in the distal pocket

Classical peroxidases oxidize many small aromatic ligands predominantly at the δ-edge of the heme, as has been reviewed by Gumiero et al.[80] Although HRP and other peroxidases catalyze the dehalogenation of phenols, no information about their halophenol binding site(s) is available. To establish if the δ-edge oxidation takes place in DHP and Mb dehaloperoxidative activity, a number of crystal soaking experiments with TCP and phenol have been carried out. In addition to wt-DHP, several mutants and very high concentration of TCP were used. This approach yielded a number of high occupancy DHP complexes for which structures were determined and are available in the Protein Data Bank: T34N•TCP, PDB 4KMW; T34N/S91G•TCP, PDB 4KN3; L100F•TCP, PDB 4KMV,[81] and a myoglobin complex Mb•phenol, PDB 3U3E.[82] Different modes of binding were observed in the two protomers present in an asymmetric part of the crystallographic unit cell consistent with high degree of flexibility of the distal pocket in DHP.

There is no correlation between the mutant peroxidase activity and crystal binding, which indicates that the observed complexes are not catalytic sites for the peroxidase function of DHP, but may be responsible for substrate inhibition observed at high TBP and TCP concentration. An internal binding site at the α heme edge, which was seen with ~0.1 occupancy in the DHP•TBP

complex (PDB 4FH6) based on anomalous diffraction, has also been observed with an occupancy of ~0.5 in one of the two subunits of the crystallographic dimer of both Y34N/S91G•TCP (PDB 4kn3) and L100F•TCP (PDB 4KMV). The higher TCP occupancy allowed us to determine that in each case the heme was in the aquomet state while the original crystals used for soaking were in the oxyferrous state. Thus, the TCP presence in soaking solutions led to heme oxidation, apparently by O_2 since H_2O_2 was not present. It may be speculated that the internal binding mode, while not catalytic, could be involved in the process of DHP oxidation/activation in the presence of halophenols. The TCP binding site near the α-heme edge is clearly not consistent with peroxidase function, so that the one-electron oxidized substrate can diffuse into solution. The α-heme-edge site may be involved in other functions of DHP,[31] or may even be an inhibitor binding site similar to the 4-BP binding site discussed above.

A second internal TCP binding site was observed at the heme β-edge with full occupancy in both protomers of Y34N•TCP (PDB 4KMW). We have recently called this the β-site because it is closer to the heme β-edge. It has a large overlap with the known inhibitor binding site as is evident in Figure 7. In Y34N/S91G•TCP, the situation is more complex. One protomer shows TCP bound in the β-site with full occupancy and the other shows TCP disordered between the α- and β-sites with equal occupancies. These two binding sites partially overlap and cannot be simultaneously occupied. Structural changes induced in DHP by TCP binding in the β-site can be observed because of the full occupancy and are shown in Fig. 6.

The distal histidine binding site has similarity with the 4-BP site in that the distal histidine is forced into the solvent-exposed (open) conformation and the TCP molecule blocks the heme Fe. In addition, chlorine atom Cl4 partially overlaps with the modeled position of the active oxygen atom of Compound I, although there is no contact between TCP and the heme Fe. There are numerous contacts shorter than 3.5 Å between the Cl2, C3 and Cl4 atoms of TCP and the heme atoms.

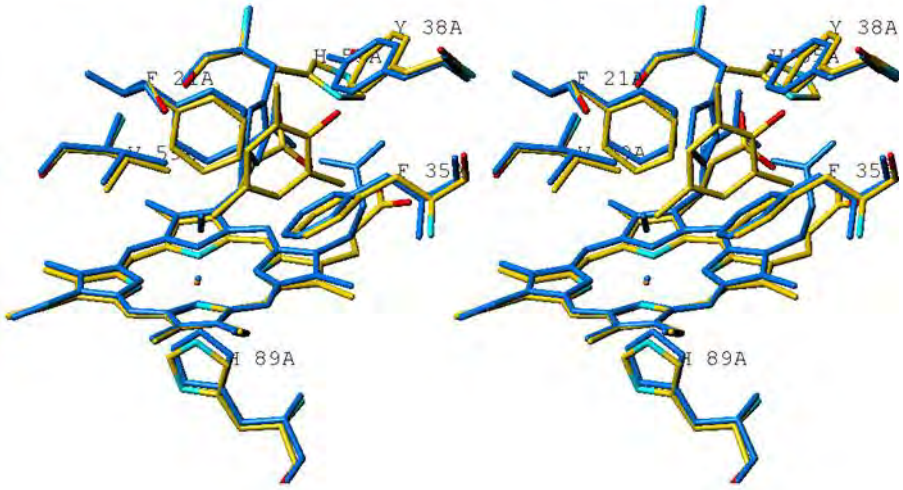

Figure 6. Stereoview of the least squares superposition of Y34N complexes with oxygen (in blue) and TCP (in atom colors). The TCP molecule pushes the heme down leading to its tilting by 6° and the movement of its edge by 0.8 Å. The Cl atom at position 4 is not in contact with the heme Fe; its position is very close to that of the distal atom of the dioxygen molecule. The TCP molecule forms contacts in a 3.1 to 3.5 Å range with the heme atoms: Cl2 atom with C1D and the D propionate, C3 with ND, C1D, C2D and C3D while Cl4 with NC, C4C and ND. Note that the PDB heme nomenclature is not the same as used here, δ-edge atoms have letter "B" in their names.

The concentrations of trihalophenols needed to observe binding in crystals are much higher than the values of K_m measured by kinetics. This is likely due to the fact that for catalysis the substrate binds to Compound I, not to the ferric enzyme and apparently this intermediate has much higher affinity for phenolic substrates. The same conclusion was reached for the classical

myoglobin from sperm whale in the study of dehaloperoxidase reaction inhibition by phenol.[82] Even though the observed in DHP external TCP binding site is inhibitory, it is not unlikely that the productive binding to Compound I or Compound II is structurally somewhat similar. First, the oxygen atom bound to Fe(IV) is strongly polarized and thus a poor hydrogen bond acceptor. It is also further from the distal histidine since the Fe-O bond is shorter. Thus the distal histidine (His55) likely is out of the distal cavity in the intermediates and can bind the hydroxyl of TCP like in the inhibitory complex. This hydroxyl forms another hydrogen bond with the hydroxyl of Tyr38. The Y38N variant of DHP has a 13-fold higher activity which can be explained by the proposed model. The absence of the hydrogen bond in Y38N reduces the electron density on the TCP hydroxyl and should facilitate electron transfer to the heme, likely through Cl4-heme contacts. These studies suggest that the emerging consensus about the role of the heme δ edge in peroxidases may apply to DHP.

There is always a concern that the binding observed is due to an artifact of the conditions (crystalline form and the use of non-aqueous solvents). Recent data demonstrating the multi-functional nature of DHP suggest that these sites may relate to non-peroxidase functions. They may also be inhibitor sites, since substrate inhibition is observed in DHP. Therefore, at this time we have no evidence that any of the binding sites is an artifact and significant evidence linking each of the sites to some aspect of known DHP function. These structures do not resolve the most important issue for peroxidase function since none of the X-ray crystallographic studies have revealed any binding near the δ-heme edge. The radical mechanism for oxidation of TCP shown by flow-EPR studies and radical trapping clearly show that there must be a significant component of external binding. Other methods (e.g. NMR) will likely be needed for in the search for an external (δ-heme edge) binding site consistent with peroxidase function. However, the X-ray crystal structure studies have revealed unique features of the hemoglobin-peroxidase that suggest it is a multi-functional enzyme. In this review we have focused only the peroxidase function. Therefore, we conclude that the internal binding sites for both 4-BP and TCP are most consistent with inhibition with regard to the peroxidase mechanism.

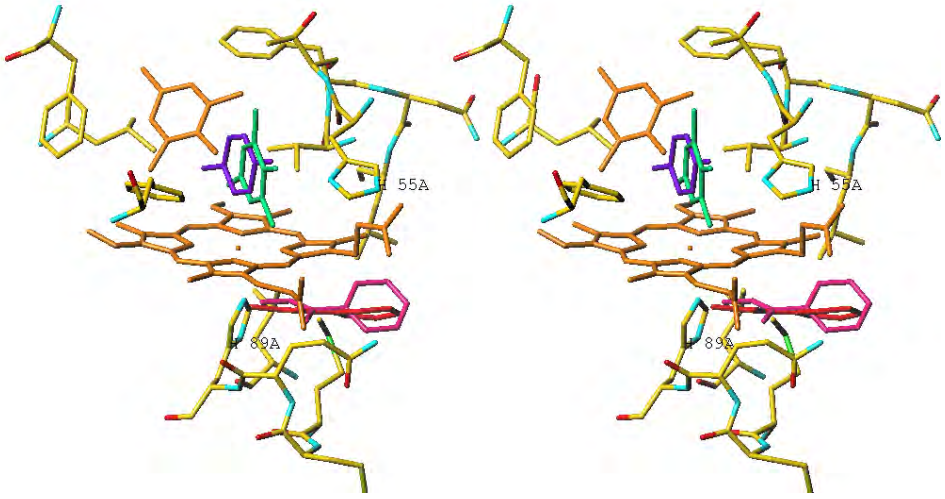

Figure 7. Substrate binding sites in DHP. Protein is shown in atom colors, the heme in brown, TCP bound in an internal site (at α-edge) is in orange, TCP in a second internal site (closest to the β-edge) site is in cyan (from PDB 4KN3), and 4-CP bound in a similar site nearest to the β-edge is in violet (from PDB 3LB3). The proximal (H98) and the distal (H55) histidines are marked. Also, the relative positions of two substrates for peroxidases are shown: benzhydroxamic acid from the complex with HRP in red (from PDB 2ATJ) and isoniazid from the complex with cytochrome C peroxidase (CcP), in purple. This figure shows that all of the crystallographically observed binding sites in DHP are in the distal cavity.

Figure 7 shows a superposition of the known internal binding sites in DHP and the binding sites in HRP and CcP. One can see that benzhydroxamic acid and isoniazid are not nearly as deeply buried as any of the internal binding sites in DHP.

2.5.4 Relationship between internal inhibitor binding and heme stability

One intriguing observation with regard to these internally bound structures is that the two highest resolution structures of DHP have 4-BP bound internally in the distal pocket. We can compare the resolution of the series of 4-halophenols bound in the distal pocket DHP(4-FP), DHP(4-CP), DHP(4-BP) and DHP(4-IP), which have resolutions of 1.56, 1.85, 1.06 and 1.76 Å (PDB 3LB4, 3LB3, 3LB2 and 3LB1), respectively (see Table 1).[65] The L100F(4-BP) mutant (PDB 4HSX) has a resolution of 1.12 Å while the resolution of the L100F protein with no ligand in the distal pocket (PDB 4HSW) is 1.22 Å.[83] The substantial increase in resolution when 4-BP binds led to the hypothesis that the specific fit of 4-BP stabilizes the globin structure. This specificity is explained, in part, by the Xe-binding cavity that is precisely where the Br atom of 4-BP binds. A corollary to this would be that the heme itself would be stabilized by the binding of 4-BP since protein denaturation in a globin is concomitant with heme loss. This hypothesis was tested using denaturants in a heme stability study. The holoprotein denatures as the heme is lost so that this is also a protein stability study that others have developed in the context of myoglobin and its mutants.[84,85] Indeed, the heme in DHP is less stable than most globins. It appears that DHP B is less stable than DHP A, which leads to the hypothesis the increased flexibility of the distal pocket leads to both greater reactivity and lower stability towards heme binding.[86] The relationship of inhibitor binding and heme stability is complicated by the fact that 4-BP also acts as a denaturant at concentrations > 3 mM and then synergistically with either urea of guanidinium hydrochloride at somewhat lower concentration. However, at modest concentration [4-BP] < 1 mM, there is an opposite effect in which 4-BP stabilizes the heme against urea denaturation.

2.6 Deactivation of DHP

In the above discussion of the DHP activity there has been an undercurrent of another important theme in DHP: the inactivation of the protein. All heme proteins are sensitive to degradation by H_2O_2, but DHP appears to be more sensitive than members of the C*c*P family. In fact, DHP is more sensitive than typical globins such as SWMb. Of course, SWMb is very poor peroxidase to begin with, but the point is that there is relatively little deactivation of SWMb compared to DHP. We can summarize the observations as a hypothesis for the inactivation of globin peroxidases: the higher the turnover of the peroxidase, the greater the rate of inactivation. This hypothesis has a corollary in the pH dependence of the kinetics. We have already seen that as the pH is lowered the apparent initial rate increases, but the final concentration of product decreases.[87] This is because the competing reaction leading to alteration of the heme also accelerates as the pH is lowered. Thus, pH lowering has a favorable effect on the kinetics, but that effect does not make DHP a better enzyme.

The hypothesis that the higher the turnover of the peroxidase leads to a greater rate of inactivation has been explored in DHP using a series of Tyr→Phe variants. DHP A has 5 tyrosines (Y16, Y28, Y34, Y38, and Y107), while DHP B has one fewer tyrosine since position 34 is naturally an asparagine in this isoform. The location of the radical in DHP A is pH-dependent, with the dominant radical attributed to Tyr34 (pH 7) and the secondary to Tyr38 (pH 5), with radical character forming on Tyr28 when both of these residues are replaced with phenylalanines. In DHP B, the radical is initially formed on Tyr38 due to the natural substitution of tyrosine to an asparagine at position 34 in the B isoform; radical character on Tyr28 is also observed at longer times. Mutagenesis studies also demonstrated that replacement of the tyrosines at both positions, 34 & 38, led to Compound I being observed on the stopped-flow timescale. While the Compound I forming variants all exhibited vastly greater peroxidase activity (~8-12 fold greater) when compared to the native DHP isoforms, they were also nearly two orders of magnitude more rapidly

inactivated by heme bleaching in the absence of substrate. This led to the conclusion that the formation of tyrosyl radicals in Compound ES is a protective mechanism against heme bleaching caused by Compound I formation in the absence of substrate. Essentially, DHP employs sacrificial substrates in the form of tyrosines that prevent irreversible heme bleaching by leading to Compound RH formation in the wild-type isoforms.

These observations beg the question: what is the structure of Compound RH? This has been a vexing problem since Compound RH is formed under conditions where there are usually a mixture of products and protein in various states of deactivation. It is known that the heme in Compound RH has a Soret band at 411 nm, which is a distinguishing feature (WT metaquo DHP has λ_{max} = 407 nm, oxyferrous DHP has λ_{max} = 418 nm, and Compound II has λ_{max} = 420 nm).[33] Thus, we can observe formation of Compound RH in a stopped-flow or benchtop mixing experiment using UV-vis spectroscopy. Using HPLC, a heme crosslinked to the protein has been observed under these conditions,[71] although tryptic digests have yet to be performed to conclusively determine the amino acid residues involved. However, attempts to obtain resonance Raman spectra or X-ray crystal structures of the H_2O_2 treated form of DHP have not resulted in any definitive result. Thus, we have no conclusive information. However, it is reasonable to assume that Compound RH is an end point for the tyrosine radicals formed during turnover in the absence of substrate. When substrate is present, the tyrosyl radicals are used in the oxidation of trihalophenol and less RH is formed. This follows from the fact that the substrate can by oxidized instead of the protein/heme and thus the substrate in essence plays a protective role vis-a-vis formation of Compound RH. Many of these same issues exist in other peroxidases, but those peroxidases are apparently less sensitive to degradation and therefore, this has not been as crucial an issue. However, one may surmise that certain crosslinks found to occur naturally in peroxidases may be part of a protective role that the protein can play.

2.7 The multifunctional nature of DHP - beyond pure peroxidase activity

Given that Compound I has been implicated in the mechanisms of other hemoproteins of vastly different enzymatic functions (i.e., cytochrome *c* oxidase, cytochrome P450 monooxygenase, human indoleamine 2,3-dioxygenase, the fungal peroxygenase *Aae*APO, horseradish peroxidase, and prostaglandin endoperoxide synthase), there exists the possibility that the hemoglobin-dehaloperoxidases exhibit additional enzymatic activities. One such activity, peroxygenase, has recently been identified in DHP using haloindole substrates.[31] In that study, the evidence strongly suggest that DHP is capable of peroxygenase activity that is similar to that observed for the peroxide shunt pathways of both P450 monooxygenase and indoleamine 2,3-dioxygenase. In this mechanism, hydrogen peroxide binds to the heme Fe and forms the high valent ferryl intermediate, followed by oxygen atom transfer to the substrate. This peroxygenase activity is observed to some extent in other heme proteins, but it is efficient in DHP and it appears specific for haloindoles that are also known to exist in the benthic ecosystems within which *A. ornata* inhabits. Moreover, the peroxygenase activity was observed to be initiated from either the ferric or oxyferrous states, with the enzyme returning to the oxyferrous state upon completion of its activity due to an unusual product-driven oxidase reaction that ultimately forms indigo derivatives as products. The fact that the peroxygenase activity is initiated from, and returns to, the oxyferrous state is likely related to the primary function of DHP of serving as an oxygen transport protein, yet also highlights the plasticity of the DHP active site for supporting multiple enzymatic functions.

3. CONCLUSION

DHP has proven to be a true peroxidase enzyme capable of the oxidative dehalogenation of chlorinated phenols. At the fundamental level, DHP introduces a new paradigm for peroxidases since it provides a means to begin to explore the evolutionary origins of the peroxidase family. Since globins have the longest known genealogy that extends back 1.8 billion years, the observation of peroxidase activity in a globin gives us a glimpse of how peroxidase activity may

have originally come into being. We do not mean to suggest that DHP is an evolutionary remnant, but rather that DHP serves as a model for our thinking about how protein function has evolved. In general, intracellular peroxidase activity that generates reactive oxygen species is thought to be unfavorable. However when environmental pressure to detoxify brominated or chlorinated compounds in marine ecosystems appeared with the emergence of species producing these toxins, a relatively inefficient, substrate-activated peroxidase was needed, thus the higher globin peroxidase activity observed in DHP evolved. The diverse range of functions exhibited by DHP highlights the versatility of this heme protein. The trade-off in being a more versatile enzyme is that DHP exhibits catalytic efficiencies below its monofunctional counterparts, albeit far greater than what is noted as side reactivity in other systems. This increased versatility at the expense of high catalytic efficiency is likely attributable to the nature of the DHP active site in that it lacks the additional structural features normally observed in more specialized systems that tune the protein towards one specific discrete function. Thus, DHP provides a platform for probing in detail how Nature evolved structure-function relationships in ancestral multifunctional systems of competent catalytic activity from lower organisms to achieve the complexity necessary for specialization of function and increased single-substrate activity observed in the monofunctional systems of higher organisms. **More critically, however, the lack of active site specialization enables DHP to catalytically degrade a much wider range of chlorinated substrates than the more specialized systems, highlighting the potential for DHP as a bioremediation agent effective against a broad range of chlorinated compounds that is beyond the reach of other enzymes due to the multifunctional nature of DHP.**

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Table 1. Crystal structures of DHP deposited into the PDB, arranged from the most recent to the initial structure.

PDB Accession No.	Ligand	Mutant	Date of Deposition
4JYQ	CO		4/14
4KJT		L100F	2/14
4JAC		T56S	11/13
4GZG	CO		9/13
4KMV	TCP	L100F	9/13
4KMW	TCP	Y34N	9/13
4KN3	TCP	Y34N/S91G	9/13
4HSW		L100F	5/13
4HSX	4BP	L100F	5/13
4FH6	TBP	10% DMSO	3/13
4FH7	TBP	20% methanol	3/13
4ILZ	TBP	10% 2-propanol	3/13
4DWT	CO		2/13
4DWU	CO		2/13
3OK5	4BP	H55D	9/11
3ORD	Xe		9/11
3OJ1		H55D	8/11
3O7N		V59W	7/11
3MOU	Xe		4/11
3MYM		M86E	4/11
3MYN		M86D	4/11
3LB1	4IP		11/10
3LB2	4BP		11/10
3LB3	4CP		11/10
3LB4	4FP		11/10
3K3U		V59W	11/10
3KUN	CN		6/10
3KUO	CN		6/10
3IXF		B isoenzyme	5/10
3DR9		Deoxy form	1/09
2QFK		Ferric form	7/08
2QFN		Oxyferrous form	7/08
1EWA	4IP		5/00
1EW6			5/00

Ligand abbreviations: TCP - 2,4,6-trichlorophenol; TBP – 2,4,6-tribromophenol; 4IP – 4-iodophenol; 4BP – 4-bromophenol; 4CP – 4-chlorophenol; 4FP – 4-fluorophenol .